Pyrex Journal of Medicinal Plant Research

Pyrex Journal of Medicinal Plant Research
Vol 2 (2) pp. 8-20 July, 2016

http://www.pyrexjournals.org/pjmpr

Full Length Research Paper

Effect of Selected Plant Parts Extracts on Liver Injuries Induced by CCl4 *in vitro*

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Accepted 28th June, 2016

Abstract

The objective of this study is to highlight the potential effects of selected plant parts extracts [Sweet violet blossoms (SVB), marjoram leaves (ML), red onion skin (ROS) and orange peel (OP)] individually or to work together to improve the liver injuries induced by CCl4 in vitro. Fish liver homogenate and human lymphocytes cultures were used as *in vitro* biological model systems. The data indicated that CCl4 induced many adverse cytotoxic, immunotoxic and genotoxic effects including lysosomal and mitochondrial dysfunctions, cell membrane integrity and decreasing in protease activity in fish liver homogenate and DNA damage in human lymphocytes cultures. Co-treatment of liver homogenate with CCl4 and the tested selected plant parts extracts as well as their mixture by concentration 0.75% exhibited many therapeutic effects through decreasing the rates of all those adverse effects. That decreasing rates in different toxic effects was depending on the type of the plant parts applied. The highest therapeutic effect was recorded for the mixture of the selected plant parts extracts (OP+SVB+ROS+ML by equal parts) followed by ROS, ML, OP and SVB, respectively. Such data indicated that there has been considerable interest in the role of complementary and alternative medicines for the prevention/treatment of liver injury induced in humans by many environmental toxins.

Keywords: Fish liver homogenate, human lymphocytes, toxicity, sweet violet blossoms, marjoram leaves, onion skin, orange peel.

INTRODUCTION

Liver diseases are a major problem throughout the world (Lawrence and Emmet, 2012). Many environmental toxins cause liver injury to humans, and despite new advances in hepatology, the treatment for liver diseases does not resolve the problems caused by these toxins (Elhassaneen and Abd Elhady, 2014; Fayez, 2016). Furthermore, despite the increasing need for agents to protect the liver from damage, modern medicine is costly and associated with multiple side effects resulting in patient non-compliance, subsequently lacks a reliable liver liver protective drug (Minjun *et al.,* 2015). Therefore, there has been considerable interest in the role of complementary and alternative medicines for the treatment of liver diseases.

Many of authorities and academic centers of research pay more attention towards the area of natural bioactive compounds called phytochemicals (phyto is Greek for plant). It is differ from vitamins and minerals in that they have known nutritional value. Some are antioxidants, protecting against harmful cell damage from oxidation (Monser, 2015). Others perform different functions that help prevent cancer (Tanaka *et al*., 1993). Scientists have identified thousands of phytochemicals, including flavonoids, glucosinolates (isothiocyanates and indoles), phenolic acids, phytates, and phytoestrogens (isoflavones and lignans), in vegetables, fruits, grains, legumes, and other plant sources.

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A vast variety of phytochemicals that are present in the daily human diet have been found to possess substantial antimutagenic and anticarcinogenic properties (Surh, 2003). The chemopreventive effects of the majority of edible phytochemicals are often attributed to their antioxidative or anti-inflammatory activities. Besides the edible chemopreventives in vegetables, fruits, herbs, and spices, some phytochemicals in diverse plants also have other beneficial health effects such as anti-obesity, lipid-lowering, and/or antidiabetic properties (Surh, 1999 and Sayed Ahmed 2016).

Around the world, the sources of phytochemicals are numerous and varied. These sources include herbs, spices, vegetables, fruits, food processing by-products etc. Amongst of the herbs, sweet violet (*Viola odorata* L.) found wild in many regions of the world, e.g. South and eastern Africa, South America, France, Italy, Australia and New Zealand, cultivated all over the world including Egypt as an ornamental plant. The part used including whole plant, leaves, roots and flowers. The phytochemical screening was carried out the sweet violet successive series extract, showed the presence of useful bioactive compounds including alkaloids, flavonoids, tannins, phenolic compounds, saponins, sterols and triterpenoids (Muhammad and Saeed, 2011; and Muhammad *et al.,* 2012). Flowers emetic, diaphoretic, febrifuge used in bilious problems, lung troubles, cough, kidney and liver diseases and also used to flavor breath fresheners (Vishal *et al*., 2009; Elhassaneen *et al.,* 2012; Abd El-Fatah, 2013). Also, Marjoram, sweet "*Origanum marjorana* L.", is a perennial herb cultivated in many countries all over the world including Egypt. It has an extensive use for culinary purposes, as well as in medicine (Chevallier, 1996). Marjoram bioactive compounds include essential oil components particularly *cis*-sabinene hydrate and *cis*-sabinene hydrate acetate which accounted for approximately 68.5% of the oil, Phenolics especially flavonoids are suggested as being essential bioactive compounds providing health benefits (El-Ghorab *et al.,* 2004; El-Safty, 2008). The pharmacological actions include antioxidant, antiviral, immunostimulating and immunomodulating activities (Economou *et al*., 1991; Kintzios *et al.,* 2002; Ninfali *et al.,* 2005).

Processing of fruits and vegetables may results in high amounts of waste materials such as peels, seeds and stones. Disposal of these materials usually represents a problem that is further aggravated by legal restrictions. Some major source of food wastes are orange *(Citrus sinensis* L.) and onion (*Allium cepa* L.), some of the most popular vegetables and fruits. The major by-products resulting from industrial peeling of onion bulbs are brown skin, the outer two fleshy leaves and the top and bottom bulbs (Schieber *et al*., 2001). The outer dry layers of onion bulbs, which are not edible and removed before processing, have been shown to contain a wide spectrum of polyphenolic components (Singh *et al*., 2009).

Some of these phenolics have been shown to possess strong antioxidant activity (El-Wazeer, 2011), protect against DNA damage induced by benz(*a*)pyrene (Huosein, 2011) and exhibited potential protective effects against acrylamide-induced cytotoxicity and immunotoxicity in primary liver cell cultures (Elhassaneen and Abd Elhady, 2014). Due to the large amounts of citrus being processed into juice, a considerable by-product industry has evolved to utilize the residual peels and other components. Citrus peels are a rich source of many bioactive compounds including fiber-pectins and flavonoids (Askar and Treptow, 1998). Flavonoids are found to possess high antioxidant activity and demonstrated many health protecting effects (Pier-Giorgio, 2000). Thus, new aspects concerning the use of agro-industrial wastes/by-products for further exploitation on the production of extracts rich with bioactive compounds have gained increasing interest because these are high-value products and their recovery may be economically attractive. Although the using of such plant parts in different medicinal applications is still in debates because of potential toxicological points of view. Therefore, the objective of this study is to highlight the potential of selected plant parts extracts (Sweet violet, marjoram, onion skin and orange peel) individually or to work together to improve the liver injuries induced by CCl4 *in vitro*.

MATERIALS AND METHODS

Materials

Selected plant parts*,* Orange "*Citrus sinensis* L." peel (OP), Sweet violet "*Viola odorata* L." blossoms (SVB), Red onion " *Allium cepa* L.*"* skin (ROS) and Marjoram "*Origanum majorana"* leaves (ML) samples were obtained from a local supermarket, Cairo, Egypt. Bolti fish (*Tilapia nilotica*) one year age were collected from the Nile River, Egypt by arrangement with some fisherman's and transported to the laboratory in 10-gallon plastic trash cans. Fish were fed and kept in conditions such as mentioned by Elhassaneen *et al.,* (2014).

Carbon tetrachloride (CCl_4, 10% liquid solution), dimethyl sulfoxide (DMSO) and Folin-Ciocalteu reagentbwere purchased from Sigma Chemical Co. (St. Louis, MO, USA). All organic solvents and other chemicals were of analytical grade were purchased from El-Ghomhorya Company for Drugs, Chemicals and Medical instruments Trading, Cairo, Egypt.

Preparation of selected plant parts extracts

After arriving of the selected plant parts samples, they

were prepared for drying process by manual sorting and washing. The drying process has been carried out using 55 ^{0}C under vacuum until arriving by the moisture in the final product to about 8%. The dried selected plant parts were used for extracts preparation according to the method of Amin *et al.,* (2004) with some modifications. In brief, the selected plant parts were chopped separately into small pieces, air dried at room temperature then homogenized in electric Blender. The blended dried were dissolved in methanol (80%, v/v) as the following: Twenty grams from dried plant plus 180 ml methanol were homogenized and transferred to a beaker and stirred at 200 rpm in an orbital shaker (Unimax 1010, Heidolph Instruments GmbH & Co. KG, and Germany) for 1 h at room temperature. The extract was then separated from the residue by filtration through What man No. 1 filter paper. The remaining residue was re-extracted twice, and then the two extracts were combined. The residual solvent of methanol extract was removed under reduced pressure at 40°C using a rotary evaporator (Laborata 4000; Heidolph Instruments GmbH & Co. KG, Germany).

Measuring of the cytotoxic effects of selected plant parts extracts and their mixtures using the fish liver homogenate

Preparation of Bolti fish liver homogenate

Bolti fish (*Tilapia nilotica*) liver homogenate were prepared according to the method mentioned by Elhassaneen, (1996) with some few modifiboltiions. Briefly, Bolti fish were anesthetized in tricaine methane sulfonate (MS-222; Sigma Chemical Co., St. Louis, MO) and weight, length, and sex was recorded. Livers were excised to a 60 × 15 mm petri dish (Baxter Healthcare Corp., McGaw Park, IL) containing Hank's Balanced Salt Solution (HBSS; Sigma Chemical Co.). Other tissues unless livers were cut away and the HBSS were removed. The livers were minced with a sterilize scissors and resuspended in RPMI-1640 (Sigma Chemical Co.) adjusted to 330 mOs/kg and supplemented with 25 mM HEPES buffer, 2 mM L(+)glutamine, 100 IU/ml penicillin, 100 g/ml streptomycin and 10% fetal Calf serum (FCS; all from Sigma Chemical Co.) to give a concentration (10 mg protein/ml).

Cytotoxicological studies

Determination of the ideal concentrations of the selected plant parts extract for applying in co-treatment with CCl4

Liver homogenate of Bolti fish were seeded at 100 µl homogenate (1 mg protein)/well of 96 flat tissue culture plate. 100 µl of RPMI-1640/FCS growth medium was added to each well. Seven tenfold dilutions of CCl_4 were prepared and 100 □l of different dilutes were added to each well (as positive control replicates). Mixture of selected plant parts extracts by equal amounts.were prepared in a concentrations (0.25-1.00%) and 100 µl of different dilutes was added to each well (as treated replicates). All plates were incubated at 27 ^{0}C for 4 h in the presence of 5% CO_2 tension. The plates were prepared for NR assay such as described in material and methods.

Effect of selected plant parts methanolic extract on the cytotoxicity of CCl4 in liver cells

Liver homogenate of Bolti fish were seeded at 100 □l homogenate (1 mg protein)/well of 96 flat tissue culture plate. 100 □l of RPMI-1640/FCS growth medium was added to each well. Seven tenfold dilutions of CCl_4 were prepared and 100 □l of different dilutes were added to each well (as positive control replicates). Selected plant parts extracts were prepared in a concentration (0.75%) and 100 □l of different dilutes were added to each well (as treated replicates). All plates were incubated at 27 □C for 4 h in the presence of 5% CO_2 tension. The plates were prepared for NR assay such as described in material and methods.

Cytotoxicity assays

Neutral red (NR), Methyl tetrazolium (MTT) and Crystal violet (CV) assays which determined the lysosomes activity, mitochondrial activity, and cell membrane integrity of liver cells, were assayed sych as described by Borenfreund and Puerner, (1984), Borenfreund and Babich, (1988) and Saotome *et al*., (1989), respectively.

Genotoxicological studies

Preparation of Human lymhocyte cells

Human lymhocyte cells were isolated according to Boyum, (1968) from whole blood by ficoll separating solution (Sigma Chemical Co.); and the cells were washed by medium of TGD of DNA. The isolated human lymphocytes were incubated with methanolic extracts of formulae and its components in TGD of DNA and comet assay medium for 2h; the viability of the cells was determined by trypan blue 1 ml (2×10^6 lymphocytes) from the stock of suspended cells in medium was transferred to 1.5 ml ependorf tube and completed to 1.5 ml with the medium. Viability of treated cells was measured after 2 h of treatment with plant parts extracts. The treated cells (0.7 ml) was transferred into 15 ml falcon tube for comet assay.

Cell viability assay

The cytotoxic effect was demonstrated by measuring cell viability by trypan blue exclusion staining at the end of incubation period according to the method of (Boyum, 1968).

The comet assay or single cell gel electrophoresis

This technique permits the detection of single strand breaks and alkali-labile site such as mentioned by Singh *et al*., (1988) and modifiboltiion was done by Hassab-Elnabi, (1996).

Statistical analyses

All experiments of cytotoxicity were performed at least three times, using four wells for each concentration of tested agent. Data for the dose-response cytotoxicity curves were presented as the arithmetic mean±SD. Comparative cytotoxicity of tested extracts i.e. the concentration of tested extract needed to reduce absorbance of the NR, MTT and CV by 10% (NR_{90}, MTT_{90} and CV_{90} values) and by 50% (NR_{50}, MTT_{50} and CV_{50} values) were computed by linear regression analysis of the data as percentage of control versus the logarithmic concentration of the tested extract (Saotome *et al*., 1989).

RESULTS AND DISCUSSION

Determination of the ideal concentrations of the selected plant parts extract for applying in co-treatment with CCl4

Bolti fish liver cells homogenate were incubated with serial dilutions of CCl4 alone and in co-treatment with mixture of the selected plant parts methanolic extract by different concentrations (0.25-1.0%). Neutral red (NR) assay which determined the lysosomal activity was used to make a dose response toxicity of CCl4. The data were standardized by expressing absorbance data in the presence of each mixture concentration as a percentage of that in the control medium. Such as shown in Figure (1), the absorbance measurements of NR assay (as % of control) were 17.51-104.78, 41.30-104.09, 50.65-107.66, 58.84-113.83 and 60.32-114.74 for the CCl4 alone and co-treatment with mixture of the selected plant parts ethanolic extract by different concentrations (0.25, 0.50, 0.75 and 1.0%), respectively.

Such data indicated that CCl4 induced highly adverse cytotoxic effects including lysosomal dysfunctions of liver cells which expressed by NR assay. Co-treatment of liver cell with CCl4 and mixture of the selected plant parts methanolic extract exhibited therapeutic effects through decreasing the cytotoxic effect. That decreasing in different cytototoxic effects was depending on concentration of the mixture applied. The therapeutic effect was gradually increased with the increasing of the mixture applied up to 0.75 % and relatively established afterthat. Such data with take the economical aspects in our considerations recommended the mixture of the selected plant parts methanolic extract by the concentration 0.75% to be the ideal concentration for application in different toxicological and therapeutic studies.

Effect of selected plant parts methanolic extracts on the cytotoxicity of CCl_4 in liver cells

Such as mentioned before Bolti fish liver cells homogenate were used as an experimental tool for studying the toxic effects of some selected plant parts and their mixture. For NR, MTT, and CV assays which determined the lysosomes activity, mitochondrial activity, and cell membrane integrity of liver cells, the data were standardized by expressing absorbance data in the presence of each extract as a percentage of that in the control medium. Table (1) and Figure (2) represent typical NR, MTT and CV assays in Bolti liver cells homogenate exposed to CCl4 and CCl4 plus the four selected plant parts extract OP, SVB, ROS,ML as well as their mixture.

The absorbance measurements of assays (as % of control) were 17.51-104.78, 32.89-109.67, 29.89-108.45, 56.76-111.67, 51.99-109.54 and 59.20-112.61 (for NR); 22.73-106.07, 34.60-112.05, 32.36-111.24, 59.58-115.25, 57.50-113.72 and 60.32-115.75 (for MTT); and 29. 56-110.79, 49.10-115.14, 41.98-116.47, 62.10-116.18, 57.53-112.81 and 63.07-119.11 (for CV) for the CCl4 and CCl4 plus the four selected plant parts extract OP, SVB, ROS,ML as well as their mixture, respectively. Such data indicated that CCl4 induced many adverse cytotoxic effects including lysosomal and mitochondrial dysfunctions and cell membrane integrity of liver cells which expressed by NR, MTT and CV assays, respectively. Co-treatment of liver cell with CCl4 and the tested selected plant parts extracts as well as their mixture exhibited therapeutic effects through decreasing the all of the different cytotoxic effects. That decreasing in different cytototoxic effects was depending on the type of the plant parts applied. The highest therapeutic effect was recorded for the mixture of the selected plant parts extracts followed by ROS, ML, OP and SVB, respectively. The highest therapeutic effects recorded by the mixture of the selected plant parts extracts could be attributed to the antagonism effects as the result of different phytochemicals categories including (Schieber *et al.,* 2001; Sayed Ahmed, 2016). Also, it could be easily concluded that, NR assay is more sensitive to all tested under this invessdtigation than others assays.

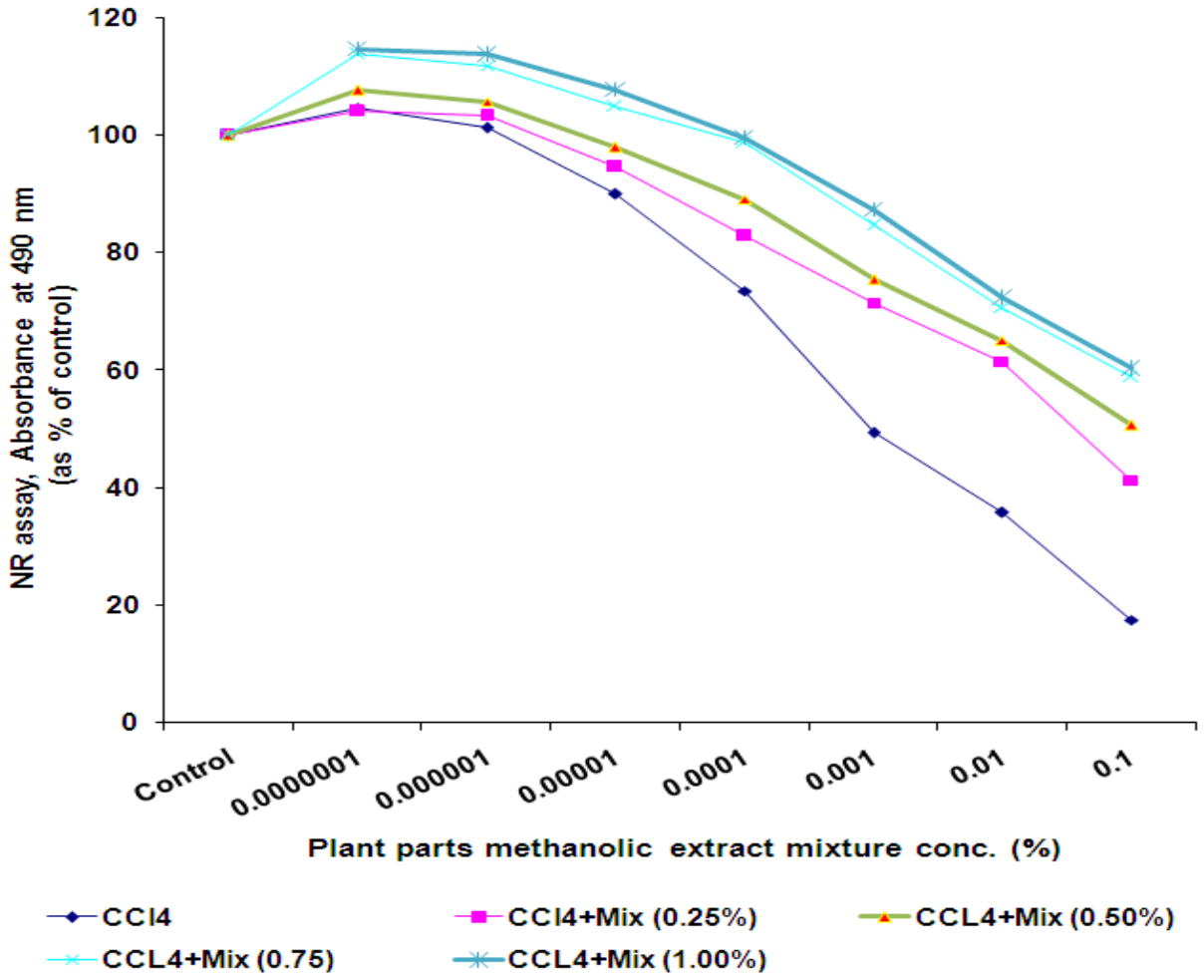

Figure 1. Effect of mixture selected plant parts methanolic extract on the cytotoxicity of CCl4 in liver cells as determined by
neutral red (NR) assay *
* CCl4, carbontetrachloride; Mix, mixture of orange peel, sweet violet blossoms, red onion skin and marjoram leaves extracts by
equal parts

These data are in agreement with that obtained by and Elhassaneen (1996) who studied the cytotoxic effects of some organic and inorganic toxic chemicals by using isolated fish hepatocytes and found that the absorbance measurements for the NR assay is more sensitive than MTT and CV assays. Also, Borenfreund and Babich (1988) who studied the cytotoxic effects of some toxic chemicals and drugs used for cancer chemotherapy by using of fibroblast cell line found that the absorbance measurements for the NR assay, as noted earlier, about twice those for the MTT assay. Furthermore, Ahmed, (2010) found that cytotoxic effects assayed by NR, MTT, and CV assays for different synthetic and natural food additive indicated that the highest adverse cytotoxic effects were recorded for synthetic additives i.e. Karate snacks followed by loleta and potato chips, respectively. A very little effects induced by natural extracts, curcumin and beet roods.

Studying the initial and midpoint toxicity of the selected plant parts methanolic extract towards liver homogenate of Bolti fish

To analyze the cytotoxicity data it was necessary to determine the exposure concentration to tested CCl4 and CCl4 plus the four selected plant parts extract OP, SVB, ROS, ML as well as their mixture causing initial toxicity (NR_{90}, MTT_{90}, CV_{90}, GA_{90}, and PA_{90} values) and those

Table 1. Effect of selected plant parts methanolic extract on the cytotoxicity of CCl4 in liver cells as determined NR, MTT and CV assays*

Conc. (%)	ABS (% of control)					
	CCl4	CCl4+ plant parts methanolic extract**				
		OP	SVB	ROS	ML	Mix
Neutral red (NR) assay						
Control	100	100	100	100	100	100
0.0000001	104.78	109.67	108.45	111.67	109.54	112.61
0.000001	101.23	105.56	103.98	108.32	104.67	110.69
0.00001	90.10	97.95	98.00	101.32	98.17	103.76
0.0001	73.54	87.40	84.45	94.54	90.65	97.67
0.001	49.32	67.34	63.13	83.67	83.56	85.71
0.01	35.76	48.92	45.76	58.78	56.59	70.99
0.1	17.51	32.89	29.87	56.76	51.99	59.20
Methyltetrazollium (MTT) assay						
Control	100	100	100	100	100	100
0.0000001	106.07	112.05	111.24	115.25	113.72	115.75
0.000001	102.48	107.85	105.70	111.80	107.71	112.79
0.00001	91.21	100.08	97.58	104.57	101.02	105.73
0.0001	74.44	89.30	89.84	97.57	93.28	99.53
0.001	49.93	78.80	64.17	86.36	75.69	90.40
0.01	37.20	50.98	49.52	70.99	59.23	73.34
0.1	22.73	34.60	32.36	59.58	57.50	60.32
Crystal violet (CV) assay						
Control	100	100	100	100	100	100
0.0000001	110.79	115.14	116.47	116.18	112.81	119.11
0.000001	107.04	111.79	110.66	114.64	109.75	116.06
0.00001	95.27	103.73	102.17	107.23	102.94	108.80
0.0001	77.76	92.56	89.88	100.05	95.05	102.41
0.001	52.15	71.31	70.19	88.55	88.13	91.02
0.01	39.86	59.84	51.75	72.79	63.36	74.47
0.1	29.56	49.10	41.98	62.10	57.53	63.07

* CCl4, carbontetrachloride; OP, orange peel extract; SVB, sweet violet blossoms extract; ROS, red onion skin extract; ML, marjoram leaves extract; Mix, mixture of OP+SVB+ROS+ML by equal parts.

causing midpoint toxicity (NR_{50}, MTT_{50}, CV_{50}, GA_{50} and PA_{50} values). Such data (Table 2) were necessary to distinguish and/or for comparison amongst the all selected plant parts methanolic extract. For example, the initial and midpoint cytotoxicity value for CCl4 was recorded lowest values for NR, MTT and CV. Co-treatment of liver cell with CCl4 and the tested selected plant parts extracts as well as their mixture induced

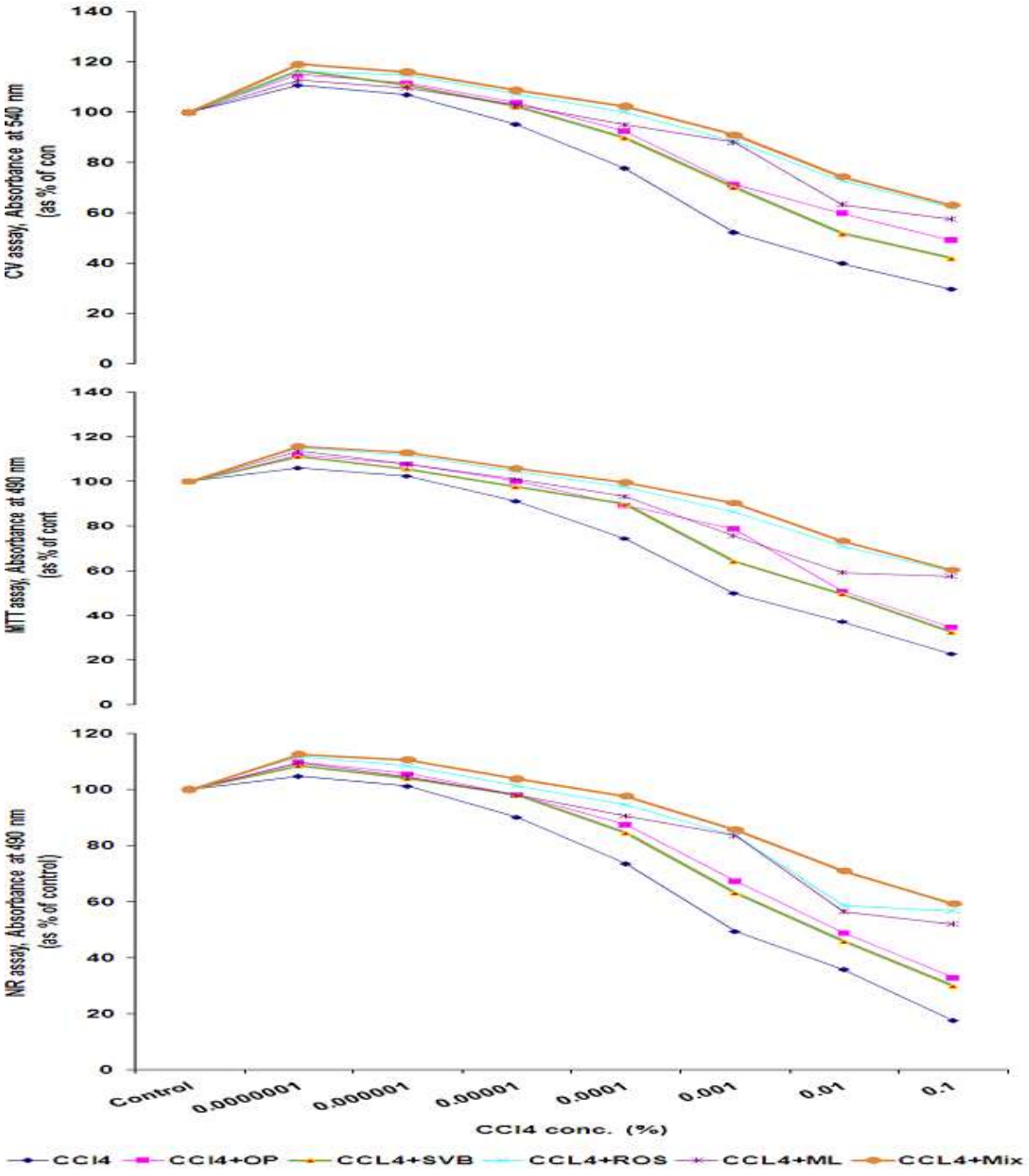

Figure 2. Effect of selected plant parts methanolic extract on the cytotoxicity of CCl4 in liver cells as determined by neutral red (NR), methyl tetrazollium (MTT) and crystal violet assays *

* CCl4, carbontetrachloride; OP, orange peel extract; SVB, sweet violet blossoms extract; ROS, red onion skin extract; ML, marjoram leaves extract; Mix, mixture of OP+SVB+ROS+ML by equal parts.

significantly reduction in the initial and midpoint cytotoxicity values i.e. exhibited therapeutic effects against CCl4 cytotoxicity. According to these data, the sequence of tested extracts for the different cytotoxicity assays were CCl4> CCl4+SVB> CCl4+OP > CCl4+ML > CCl4+ROS > CCl4+Mix.

From the above-mentioned data it could be noticed that, CCl4 induced many cytotoxic effects in liver homogenate.

These cytotoxic effects include lysosomes and mitochondria dysfunction as well as cell wall membrane integrity, which assayed by NR, MTT, and CV assays. There are some variations between the sensitivity of different cytotoxic assays. These Variations may be resulted from the difference of the idea, which each assay based on. For example, the lysosomes activity assay is based on the uptake of neutral red (NR), a supravital dye, and its accumulation in the lysosomes of viable uninjured cells (Borenfreund and Puerner, 1984). While, the mitochondria activity assay is based on the reduction of soluble yellow tetrazolium salt (MTT) to a blue insoluble MTT formazan product by mitochondrial succinic dehydrogenase (Mosmann, 1983). So, the lower sensitivity of the mitochondria in tested chemical may be due to the poor solubility of the MTT formazan product and/or lower amount of tetrazolium salt reduced by mitochondria.

Also, the tested plant parts methanolic extract induced some immunologocal effects in liver homogenate include decreasing in protein synthesis and protease activity. Such as mentioned by Neurath (1989) all of these factors play an important role in immunological functions of liver cells. The present data are in accordance with that obtained by Ragab (2003) when exposure fish liver homogenate to mish cheese biogenic amines extract.

In general, by using three cytotoxic testing i.e. NR, MTT, and CV; and one immunotoxic assays i.e. PA, we were able to demonstrate three different toxic responses as a consequence of exposure to CCl4 and Co-treatment of CCl4 with the all selected plant parts. The first type of response was the inhibition of cell division, which characterized by stabilization or slightly increase the initial count of cultured cells even with increasing the concentration of toxicant. Kocan *et al.*, (1985) attributed this type of response to the cellular dysfunction or damage. The second type of response was the cytotoxicity or cell death, which could be seen in cultures by the lower of the cells count present at the termination of the exposure period than in the beginning of the exposure to toxic substances. This response could be characterized also by a dose-response curve which is inversely proportional to the concentration of tested toxicant substances. Kocan *et al.*, (1985) demonstrated that cytotoxicity can be resulted from cells dying and/or inhibition of cell proliferation. Hormesis represented the third response which means increasing occurs in cells number over the controls at low concentrations of the toxic substance but the toxic effect does not manifest itself until a higher critical dose level is reached (Laughlin *et al.*, 1981). Previous studies of Kocan *et al.,* (1985) and Elhassaneen *et al.,* (1997) demonstrated that cytotoxicity can be resulted from cells dying and/or inhibition of cell proliferation. All of the present data are in accordance with that obtained by Elhassaneen, (1996); Elhassaneen *et al.,* (1997), Elhassaneen, (2001), Ragab (2003); Ahmed (2010), Monser (2011) and Elsabakhawy, (2011) when fish isolated/ homogenate liver cells were exposed to paper industry effluent, pesticides, heavy metals, polycyclic aromatic hydrocarbons, some of the synthetic food additives extracts, food biogenic amines extract and plant parts extract.

Effect of selected plant parts methanolic extract on the immunotoxicity of CCl4 in liver cells

The influence of selected plant parts methanolic extract on the immunotoxicity of CCl4 in liver cells which performing by protease activity (PA) assay (Figure 3). The absorbance measurements of PA assays (as % of control) were 41.04-116.33, 54.17-119.99, 50.92-120.19, 64.65-119.09, 60.68-115.06 and 65.33-122.33for the CCl4 and CCl4 plus the four selected plant parts extract OP, SVB, ROS,ML as well as their mixture, respectively. Such data indicated that CCl4 induced some adverse immunotoxic effects which expressed by PA assay. Co-treatment of liver cell with CCl4 and the tested selected plant parts extracts as well as their mixture exhibited therapeutic effects through decreasing the immunotoxic effects. That decreasing in mmunotoxic effects was depending on the type of the plant parts applied. The highest therapeutic effect was recorded for the mixture of the selected plant parts extracts followed by ROS, ML, OP and SVB, respectively. The highest therapeutic effects recorded by the mixture of the selected plant parts extracts could be attributed to the antagonism effects as the result of different phytochemicals categories including (Schieber *et al.,* 2001; Sayed Ahmed, 2016). The present data demonstrated that the dose-response curves with the NR, MTT and CV assays corresponded well to those with PA assay.

In similar studies, polycyclic aromatic hydrocarbon extracts (PAH) extracts from deep-fat frying popular Egyptian foods i.e. potato chips and tamia induced many biochemical and immunological effects in primary cultures live cells include decreasing in protein synthesis (GA) and protease activity (PA) (Elhassaneen, 2002). Such as mentioned by Neurath (1989), all of these factors play an important role in immunological functions of liver cells. Also, the present data are in accordance with that obtained by Elhassaneen *et al.,* (1997) and Elhassaneen, (2001) when exposure fish isolated liver cells to paper industry effluent, pesticides, and heavy metals. Also, Ahmed (2010) studied the influence of five synthetic and natural food additives extracted from snacks distributed in Egyptian local markets on the immunotoxicity assays which performing by protease activity (PA) of liver cells homogenate. The highest adverse effects were recorded for synthetic additives while a very little effects induced by natural extracts

Table 2. Effect of selected plant parts methanolic extract on the comparative cytotoxic effects of CCl4 in liver cells as determined by different assays

Components	NR assay		MTT assay		CV assay	
	NR_{90} *	NR_{50} **	MTT_{90}	MTT_{50}	CV_{90}	CV_{50}
CCl4	0.0000091	0.00082	0.00001	0.00082	0.000028	0.001
CCl4+ Orange peel (OP)	0.000064	0.0082	0.000082	0.01	0.000091	0.064
CCl4+ Sweet violet blossoms (SVB)	0.000064	0.0064	0.000055	0.0064	0.000082	0.01
CCl4+ Red onion skin (ROS)	0.00046	ND	0.00046	ND	0.00064	ND
CCl4+ Marjoram leaves (ML)	0.000091	ND	0.0001	ND	0.00046	ND
CCl4+Mix***	0.000064	ND	0.00082	ND	0.001	ND

* Initial toxicity: mean concentration of CCl4 or CCl4+selected plant parts methanolic extrat required to reduce absorbance by 10% to initial toxicity (NR_{90}, MTT_{90} and CV_{90}).
** Mid toxicity: mean concentration of of CCl4 or CCl4+selected plant parts methanolic extrat required to reduce absorbance by 50% to initial toxicity (NR_{50}, MTT_{50} and CV_{50})
*** Mix, mixture of OP+SVB+ROS+ML by equal parts

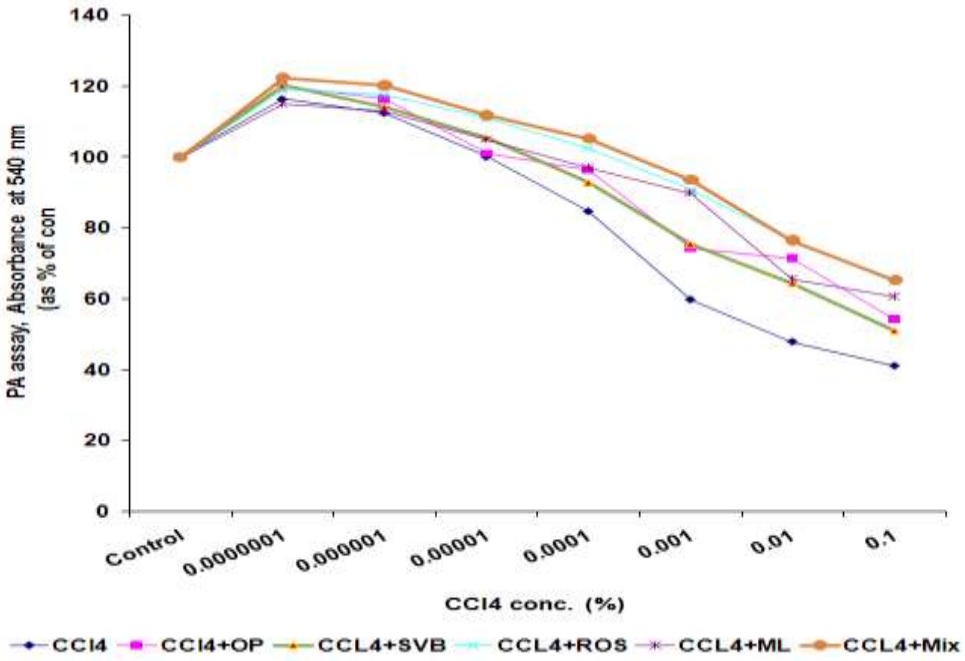

Figure 3. Effect of selected plant parts methanolic extract on the immunotoxicity of CCl4 in liver cells as determined
by protease activity (PA) assay *
* CCl4, carbontetrachloride; OP, orange peel extract; SVB, sweet violet blossoms extract; ROS, red onion skin extract; ML, marjoram
leaves extract; Mix, mixture of OP+SVB+ROS+ML by equal parts

(curcumin and beet roods).

Research has examined a variety of natural substances that demonstrate immunomodulatory potential. Immunomo-dulation is described as the ability of a nutrient, herb, or other substance to promote healthy immune function (Brown, 1996). Certain plant compounds have been shown in experimental studies to have immunostimulating properties; that is, they appear to help stimulate viral defense mechanisms by activating immune cells such as macrophages, lymphocytes (T and B-cell, and natural killer cell), and the cytokines (e.g., interleukin, interferon, and tumor necrosis factor) (Roesler *et al.,* 1991and Suresh and Vasudevan, 1994).

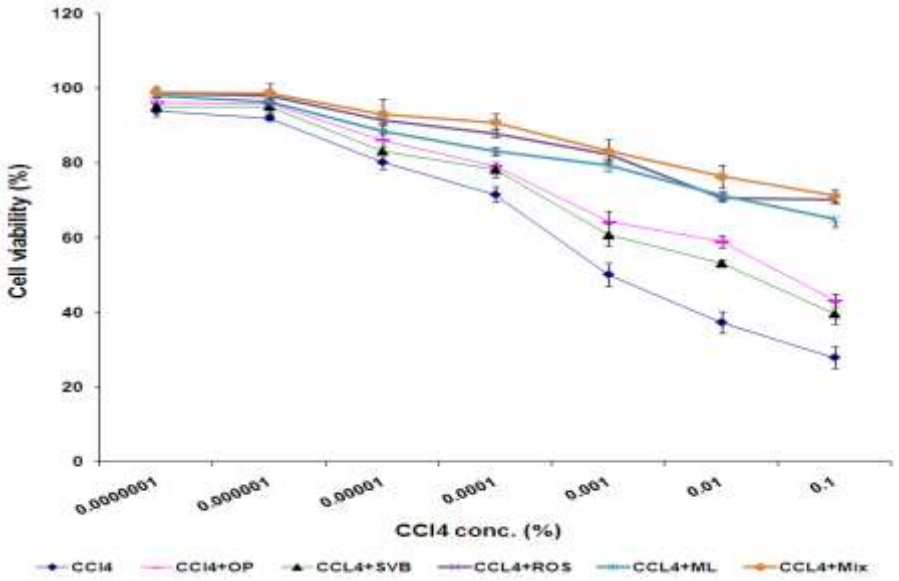

Figure 4. Effect of selected plant parts methanolic extract on the viability of lymphocytes as affected by CCl4 *

* CCl4, carbontetrachloride; OP, orange peel extract; SVB, sweet violet blossoms extract; ROS, red onion skin extract;
ML, marjoram leaves extract; Mix, mixture of OP+SVB+ROS+ML by equal parts

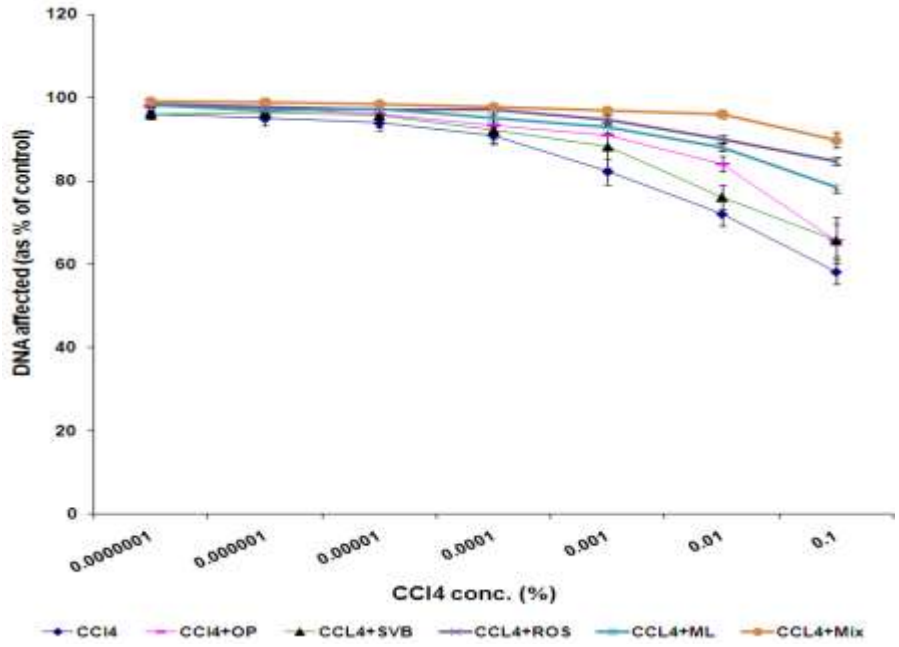

Figure 5. Effect of selected plant parts methanolic extract on the comet assay of DNA-lymphocytes as affected by CCl4 *

* CCl4, carbontetrachloride; OP, orange peel extract; SVB, sweet violet blossoms extract; ROS, red onion skin extract;
ML, marjoram leaves extract; Mix, mixture of OP+SVB+ROS+ML by equal parts

EFFECT OF SELECTED PLANT PARTS METHANOLIC EXTRACT ON THE GENOTOXICITY OF CCL_4 BY USING OF HUMAN LYMPHOCYTE CULTURES

Viability of human lymphocyte

Figure (4) shows the viability of human lymphocyte exposed to CCl4 and CCl4 plus the four selected plant parts extract OP, SVB, ROS,ML as well as their mixture. The tested dilutions of CCl4, 10^{-7} to 10^{-1}, decreased the human lymphocyte viability which were ranged 94.12 to 27.98% . The co-treatment of CCl4 with OP, SVB, ROS and ML as well as their mixture increased the human lymphocyte viability which ranged 96.18-43.24, 95.01-39.67, 98.67-70.14, 97.99-65.05 and 99.04-71.29%, respectively.n Like of differentiations in the degree of viability by the different selected plant parts methanolic extract could be attributed to the effect of their components and/or the interactive reactions. The present data are in accordance with that observed by El-Safty, (2012).

DNA damage detection by comet assay

Figure (5) shows the percentages of normal and migrated spots of DNA of CCl4 and CCl4 plus the four selected plant parts extract OP, SVB, ROS,ML as well as their mixture. The tested dilutions of CCl4, 10^{-7} to 10^{-1}, increased the percentage of total damaged spots and the normal DNA-lymphocytes (as % of control) were ranged 95.95 to 58.18%. The co-treatment of CCl4 with OP, SVB, ROS and ML as well as their mixture increased the normal DNA-lymphocytes which ranged 97.93-65.76, 96.05-65.73, 98.55-84.73, 98.3-78.45 and 99.23-89.76 %, respectively. These values were found to be highly significant ($p<0.01$) and dose dependent. All of these data are in accordance with that obtained by many previous investigations. For example, Hassab-Elnabi (1996) used human lymphocytes in studying the antigenotoxic effect of propolis and cloves with lead nitrate as a heavy metal. The comet assay has already been used in many studies to assess DNA damage and repair induced by various agents in a variety of cells *in vitro* and *in vivo* (Fairbairn *et al.,* 1995 and Tice, 1995). The test has widespread applications in genotoxicity testing, DNA damage and repair studies, environmental biomonitoring, and human pollution menitoring (Reviewed in Speit and Hartmann, 2000). A broad spectrum of DNA-damaging agents causes increased DNA migration in the comet assay: UV and ionizing radiation, hydrogen peroxide and other radical-forming chemicals, alkylating agents, polycyclic aromatic hydrocarbons, and other adduct-forming compounds, radiomimetic chemicals, and various metals (Tice, 1995). Also, Ragab (2003) showed that biogenic amines extract of Mesh cheese increased the percentage of total damaged spots of human lymphocytes. Furthermore, Ahmed, (2010) reported that some of the synthetic food additives induced much total damaged spots of human lymphocytes when compared with the natural ones. Finally, El-Sabakhawy (2011) used DNA damage percentage of human lymphocytes in studying the genotoxicity of some plant formulae and found that no DNA damage was observed.

CONCLUSION

The data of the present study indicated that using of liver cells homogenate and human lymphocytes cultures as *in vitro* biological model systems could be constitute the mile stone through open new avenues in the fields of food and hepatic toxicology through using simple, fast and accurate toxicology evaluating methods with low coasts. Co-treatment of fish liver cells or human lymphocytes with CCl4 and four selected plant parts extracts (OP, SVB, ROS, ML) as well as their mixture exhibited many therapeutic effects through decreasing the all of the different cytotoxic, immunotoxic and genotoxic effects induced by CCl4. All of the present data confirmed that there has been considerable interest in the role of complementary and alternative medicines for the prevention/treatment of liver injury induced in humans by many environmental toxins.

ACKNOWLEDGEMENTS

The authors would like to acknowledge Prof. Dr. Sobhy Hassab El-Nabi, Department of Zoology, Faculty of Science, Minoufiya University for *in vitro* experiments and lab assistance. Also, deep thanks are extended to the Research Supporting Fund (RSF), Minoufiya University, Shebin El-Kom, Egypt for the partially financial support.

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